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Cellular HSP90 (HSP86) mRNA level and in vitro differentiation of mouse embryonal carcinoma (F9) cells

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Treatment of mouse embryonal carcinoma (F9) cells with retinoic acid, an inducer of F9 cell differentiation, greatly increased the level of mRNA specific to one of the heat-shock proteins (HSP86). Experiments including the one employing differentiation-resistant mutant F9 cells suggested that the increase represents early molecular events associated with the embryonal differentiation. The increased HSP86 mRNA declined to the original level during further incubation. The presence of cyclic AMP, which stimulates conversion of the retinoic acid-induced primitive endoderm cells to parietal endoderm cells, prevented the decline. These results suggest that not only the elevation of HSP86 mRNA level represents early molecular events in F9 cell differentiation but also that sustaining the elevated level (by cyclic AMP) is associated with further differentiation of the embryonal cells.

Differentiation; Embryonal carcinoma; Heat-shock protein; HSP90; Retinoic acid; Cyclic AMP

1. INTRODUCTION

Three major groups of heat-shock proteins (HSP), proteins with mol. mass of 20–30, 68–73 and 80–90 kDa, are widely distributed throughout living organisms from bacteria to mammals, suggesting their ubiquitous roles in fundamental cellular functions. Among them, HSP with mol. mass of 68–73 kDa (HSP70) are believed to play a critical role in safeguarding the cells from damages caused by stresses such as heat shock [1,2]. On the other hand, the biological role of HSP with mol. mass of 80–90 kDa (HSP90), one of the most abundant protein species which comprises two major protein classes (HSP84 and HSP86), still remains largely unknown. A limited number of biochemical reactions known to involve HSP90 include the formation of complexes with steroid hormone receptors [3–6] or tyrosine kinases [7–11]. A suggestion was made that HSP90 is required for intracellular protein transport [12].

In order to explore the possibility that HSP90 may be involved in the molecular cascade in cellular differentiation, we examined the level of HSP90 mRNA during in vitro differentiation of mouse embryonal carcinoma (F9) cells. Here we report that the mRNA level of HSP86, a major class of HSP90, increased significantly at the very early stage of F9 differentiation triggered by retinoic acid, but the level declined sharply to the pretreatment level after several hours. The presence of (dibutyryl) cyclic AMP, which stimulates the conversion of primitive endoderm cells to parietal endoderm cells in the F9 embryonal differentiation lineage [13], prevented the decline and kept HSP86 mRNA at the elevated level for several days. A possible role of HSP86 mRNA in F9 differentiation is discussed.

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2. MATERIALS AND METHODS

2.1. *Materials*

Herbimycin A was kindly supplied by Dr Y. Uehara. Retinoic acid and dibutyryl cyclic AMP were purchased from Sigma (St. Louis, MO). All the other agents were reagent-grade. ES medium was purchased from Nissui Seiyaku (Tokyo). Fetal calf serum was obtained from Sigma [^{32}P]dCTP and [^{35}S]methionine were purchased from ICN Biomedicals (Irvine, CA).

2.2. *Cells and cell culture*

F9 cells were supplied by Dr Y. Nishimune. Differentiation-resistant mutant cell lines (RA^r-6 and RA^r-OT) were isolated from *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-treated F9 cells as will be described [14]. The cells were cultured at 37°C in a CO_2 (5%) incubator in ES medium supplemented with FCS (10% (vol/vol)). Plastic tissue culture dishes had been coated with gelatin solution (0.4% (wt/vol)) before use.

2.3. *Isolation of a HSP90 cDNA clone (pMHS86)*

For the isolation of HSP90 clone (pMHS86), which was used as a probe for Northern hybridization to detect HSP86 mRNA, a λgt10 cDNA library was first constructed from RNA isolated from F9 cells which had been incubated with herbimycin A (0.5 μg/ml) for 6 h. Sequencing of a clone (λH707), which was derived from an mRNA species specific to at the early stage of F9 differentiation, showed that the clone contained sequences identical with part of the HSP86 gene [15], a major HSP90 member. Using λH707 as a probe, a new clone (pMHS86) with more complete HSP86 sequences including 5'-noncoding region was isolated from the mouse Okayama–Berg library. A portion of pMHS86 (total 380 bp) between *Xho*I (in the vector) and *Bgl*I (5' side of the initial ATG) specific to HSP86 was used as the probe for Northern hybridization.

2.4. *Northern hybridization*

RNAs were prepared from the cytoplasmic fraction of F9 cells (~10^7 cells) according to the method described by Wisnievski et al. [16]. Northern hybridization was performed as described [17]. RNA (5 μg) was electrophoresed through agarose gel (1%), transferred to nylon membrane filters and hybridized with pMHS86 or chicken β-actin-probe (pA1) [18] which had been labeled with [^{32}P]dCTP (ICN) by random priming [19].

2.5. *Autoradiography of proteins*

F9 cells were labeled with [^{35}S]methionine (ICN, 200 μCi/ml, 3000 Ci/mmol) for 30 min as indicated in the legend to Fig. 4. The cells were lysed and the samples were subjected to SDS-PAGE (7.5% acrylamide) [20] and autoradiography using Kodak X-OMAT films.

3. RESULTS AND DISCUSSION

Mouse embryonal carcinoma (F9) cells differentiate in vitro into primitive endoderm-like cells following exposure to retinoic acid [21]. The presence of (dibutyryl) cyclic AMP further stimulates the differentiation into parietal endoderm-like cells [13]. In order to explore the possible role of heat-shock proteins, particularly of HSP90, in embryonal differentiation, we treated F9 cells with retinoic acid and RNA was subjected to Northern blot hybridization after electrophoresis using a HSP90 probe (pMHS86) which represented 5'-non-coding region unique to the HSP86 gene. As seen in Fig. 1A, RNA isolated after a 6-h incubation with retinoic acid gave a considerably higher signal than the control RNA from untreated cells. The presence of (dibutyryl) cyclic AMP in addition to retinoic acid apparently had no effect on the retinoic acid-induced increase of HSP86 mRNA at least at this very early stage of differentiation (Fig. 1A, lane 3, and see below).

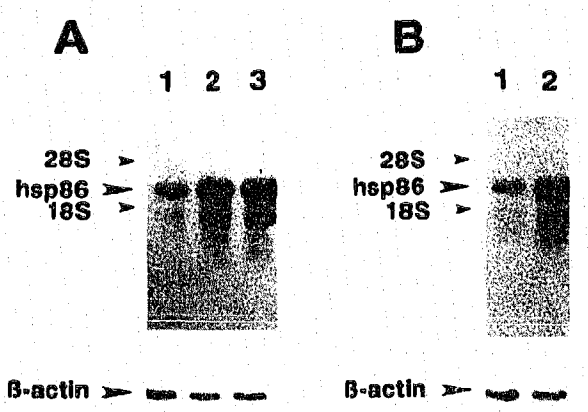

Fig. 1. Change of HSP86 mRNA levels during F9 differentiation. A. F9 cells were incubated with retinoic acid (1 μM) or retinoic acid (1 μM) plus dibutyryl cyclic AMP (0.5 mM) for 0 and 6 h. RNA was isolated from each sample (~10^7 cells) and subjected to Northern blot hybridization using ^{32}P-labeled HSP86 probe (pMHS86) or β-actin probe (pA1). The positions of HSP86 mRNA, β-actin mRNA and molecular weight markers (18S 28S ribosomal RNA) are indicated. For details, see section 2. Lane 1, control cells (0 h); lane 2, cells incubated with retinoic acid for 6 ; lane 3, cells incubated with retinoic acid plus dibutyryl cyclic AMP for 6 h. B. Same as A, but F9 cells were incubated with herbimycin A (0.5 μg/ml) for 0 and 6 h. Lane 1, control cells (0 h); lane 2, cells incubated with herbimycin A for 6 h. For details, see above and section 2.

In order to determine whether the increase of HSP86 transcripts following retinoic acid treatment represented early molecular events of F9 cell differentiation or the increase was a result of specific and direct activation of the HSP86 gene by retinoic acid, we also examined the level of HSP86 transcripts in the cells which had been treated with herbimycin A, an inhibitor of tyrosine protein kinase [22–24] and another potent inducer of F9 cell differentiation. Herbimycin A, even without (dibu-

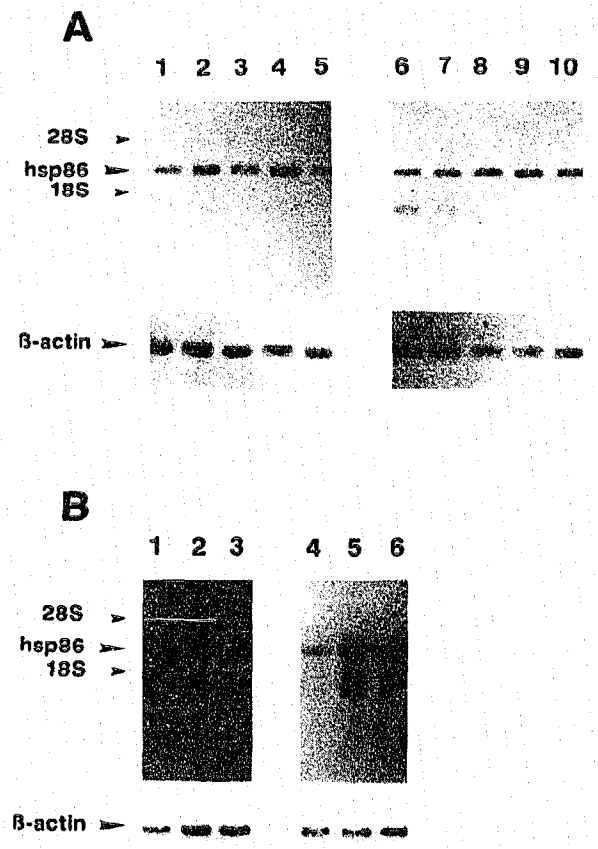

Fig. 2. HSP86 mRNA levels in differentiation-resistant F9 mutant cells. A. Differentiation-resistant F9 mutant cells (RA^r-6 and RA^r-OT) were incubated in the presence of retinoic acid (1 μM) or retinoic acid (1 μM) plus dibutyryl cyclic AMP (0.5 mM) for 0, 6 and 96 h. RNA was isolated from each sample (~10^7 cells) and subjected to Northern blot hybridization as described in the legend to Fig. 1 and section 2. Lane 1–5, RNAs from RA^r-6; lane 6–10, RNAs from RA^r-OT. Lane 1 and 6, control (0 h); lane 2 and 7, cells incubated with retinoic acid for 6 h; lane 3 and 8, cells incubated with retinoic acid plus dibutyryl cyclic AMP for 6 h; lane 4 and 9, cells incubated with retinoic acid for 96 h and lane 5 and 10, cells incubated with retinoic acid plus dibutyryl cyclic AMP for 96 h. B. Same as A, but RA^r-6 and RA^r-OT cells were incubated in the presence of herbimycin A (0.5 μg/ml) for 0, 6 and 96 h. Lane 1–3, RNAs from RA^r-6; and lane 4–6, RNAs from RA^r-OT. Lane 1 and 4, control (0 h); lane 2 and 5, cells incubated with herbimycin A for 6 h; and lane 3 and 6, cells incubated with herbimycin A for 96 h. The positions of HSP86 mRNA, β-actin mRNA and molecular weight markers (18S and 28S ribosomal RNA) are indicated. For details, see the legend to Fig. 1 and section 2.

tyryl) cyclic AMP, triggers F9 cell differentiation and converts F9 cells into cells which are very similar to parietal endoderm cells [25]. In Fig. 1B, we show the results of Northern blot hybridization with RNA extracted from herbimycin A-treated cells. It was quite clear that the level of HSP86 transcripts also increased significantly after incubation with herbimycin A. These results suggest that the increase of HSP86 mRNA levels is one of the early events associated with F9 cell differentiation rather than the result of specific activation of HSP86 gene by retinoic acid. The level of mRNA specific to another major heat-shock protein, HSP70, was not affected either by retinoic acid or herbimycin A treatment (data not shown).

To further investigate the possible association of the increase of HSP86 mRNA level with F9 differentiation, two mutant F9 cells (RA^r-OT and RA^r-6), which were resistant to retinoic acid-induced differentiation but still sensitive to herbimycin A-induced differentiation, were isolated. Detailed characteristics of these F9 mutant cells will be reported elsewhere [14]. The mutant cells were exposed to retinoic acid and RNA extracted from them was subjected to Northern blot hybridization. No apparent increase of HSP86 transcripts was observed up to 96 h incubation with retinoic acid (Fig. 2A). On the other hand, the level of HSP86 mRNA in these mutant cells increased considerably by herbimycin A treatment which led the mutant cells to normal differentiation (Fig. 2B). The basal level of the transcripts in the mutant cells without the drug treatment was almost equal to that of the parental cells (data not shown, also compare lane 1 in Fig. 1B with lanes 1 and 4 in Fig. 2B). Taken together, it seems that the early increase of HSP86 mRNA levels after exposure of the cells to retinoic acid (or herbimycin A) is a phenomenon closely associated with F9 cell differentiation, particularly that involved at the very early stage of differentiation.

After the initial rather dramatic increase, however, the HSP86 mRNA levels remained at that elevated level only transiently, having declined to the pretreatment level during further incubation (Fig. 3). Interestingly, the decline, which occurred between 12 h and 24 h after exposure to retinoic acid, was blocked when (dibutyryl) cyclic AMP was present in the culture medium. As described above, (dibutyryl) cyclic AMP is known to stimulate the conversion of retinoic acid-induced primitive endoderm-like cells to parietal endoderm-like cells in the F9 differentiation lineage. As seen in Fig. 3, the presence of (dibutyryl) cyclic AMP in addition to retinoic acid kept the HSP86 mRNA levels to be elevated as long as for 96 h, during which the cells were converted to parietal endoderm-like cells. Incubation of the cells with (dibutyryl) cyclic AMP alone did not increase the HSP86 mRNA levels over the control levels (data not shown). The decline of HSP86 mRNA levels seemed irreversible, for the addition of (dibutyryl) cyclic AMP to the culture medium after the decline (for example, addition after 48 h incubation with retinoic acid) had no effect (data not shown). Furthermore, in the herbimycin A-treated cells, in which the cells directly differentiate to parietal endoderm-like cells, no such decline of HSP86 mRNA levels was observed even after prolonged incubation (data not shown). Thus, one could speculate that sustaining the elevated HSP86 mRNA levels is necessary for or at least associated with further F9 cell differentiation into parietal endoderm-like cells.

We also examined the rate of synthesis of HSP86 gene

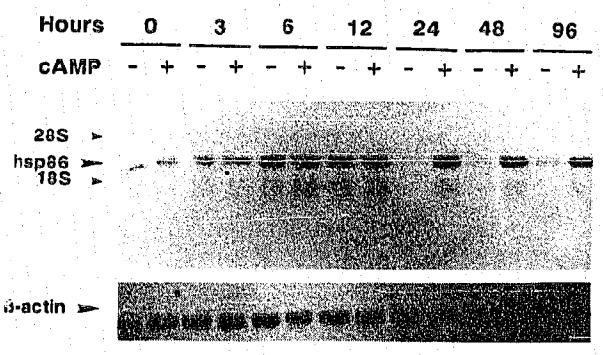

Fig. 3. HSP86 mRNA level as a function of incubation time and the effect of (dibutyryl) cyclic AMP. F9 cells were incubated in the presence of retinoic acid (1 μM) or retinoic acid (1 μM) plus dibutyryl cyclic AMP (0.5 mM). At 0, 3, 6, 12, 24, 36, 48 and 96 h, RNAs were extracted from ~10^7 cells and subjected to Northern blot hybridization as described in the legend to Fig. 1 and section 2. Time (incubation time; hours) and present (+) or absence (−) of dibutyryl cyclic AMP in the medium in addition to retinoic acid are indicated as well as the positions of HSP86 mRNA, β-actin mRNA and molecular weight markers (18S and 28S ribosomal RNA). For details, see section 2.

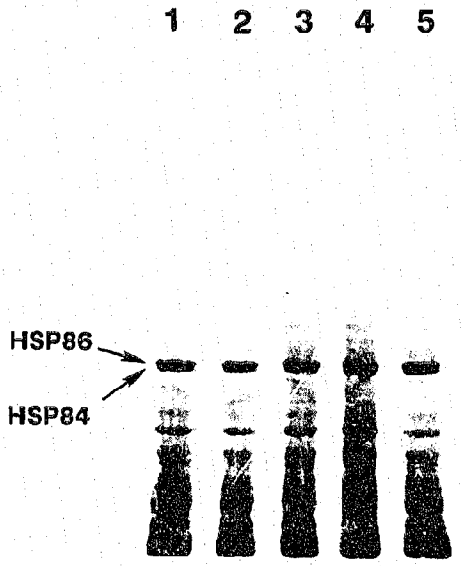

Fig. 4. Analysis of cellular proteins by autoradiography. F9 cells were incubated in the presence of retinoic acid (1 μM) or retinoic acid (1 μM) plus dibutyryl cyclic AMP (0.5 mM). At 0, 6 and 96 h, the cells were pulse-labeled with [^{35}S]methionine (200 μCi/ml) for 30 min. The cells were then lysed, and the samples were subjected to SDS-PAGE and autoradiography. The positions of HSP86 and HSP84 are indicated.

product, HSP86 protein, during F9 differentiation. In contrast to HSP86 mRNA, to our surprise, the rate of incorporation of [^{35}S]methionine into HSP86 (and HSP84) remained relatively constant during the differentiation and no effect of (dibutyryl) cyclic AMP was observed (Fig. 4). Several possibilities can be considered to explain these results. Moore et al. have recently reported that a HSP86 probe hybridized with sequences located on three separate chromosomes suggesting that the HSP86 gene comprises three HSP86 family members which may include HSP86 pseudogenes [15]. It is, therefore, possible that the increased HSP86 mRNA observed during F9 embryonal differentiation was derived from one of these genes, the transcription product of which is not translated. Alternatively, HSP86 gene expression may be regulated by an, as yet, unknown mechanism because the 5'-non-coding region of the HSP86 gene contained extraneous ATG triplets, quite an unusual feature observed in eukaryotic mRNA [15]. The other possibilities include the one which assumes a mechanism to maintain the rate of HSP86 protein synthesis relatively constant despite the considerable change in the HSP86 mRNA level. In any event, the present experiments indicated that the level of HSP86 transcripts changes in a manner closely associated with F9 cell differentiation and the presence of (dibutyryl) cyclic AMP sustains this level to be elevated which may be closely associated with the further differentiation of F9 cells from retinoic acid-induced primitive endoderm-like cells to parietal endoderm-like cells.

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REFERENCES

[1] Lewis, M.J. and Pelham, H.R.B. (1985) EMBO J. 4, 3137–3143.
[2] Welch, W.J. and Suban, J.P. (1986) J. Cell Biol. 103, 2035–2052.
[3] Sanchez, E.R., Toft, D.O., Schlesinger, M.L. and Pratt, W.B. (1985) J. Biol. Chem. 260, 12398–12401.
[4] Suhuh, S., Yamamoto, W., Brugge, J., Bauer, V.J., Riehl, R.M., Sullivan, W.P. and Toft, D.O. (1985) J. Biol. Chem. 260, 14292–14296.
[5] Catelli, M.G., Binart, N., Jung-Testas, I., Renoir, J.M., Baulier, E.E., Feramisco, J.R. and Welch, W.J. (1985) EMBO J. 4, 3131–3135.
[6] Sanchez, E.R., Housley, P.R. and Pratt, W.B. (1986) J. Steroid Biochem. 24, 9–18.
[7] Opperman, H., Levinson, A.D., Levintow, L., Vermas, H.E., Bishop, J.M. and Kawai, S. (1981) Virology 113, 736–751.
[8] Brugge, J.S., Erikson, E. and Erikson, R.L. (1981) Cell 25, 363–372.
[9] Lipsich, L.A., Cutt, J.R. and Brugge, J.S. (1982) Mol. Cell. Biol. 2, 875–880.
[10] Adkins, B., Hunter, T. and Sefton, B.M. (1982) J. Virol. 43, 448–455.
[11] Courtneidge, S.A. and Bishop, J.M. (1982) Proc. Natl. Acad. Sci. USA 79, 7117–7121.
[12] Koyasu, S., Nishida, E., Kadowaki, T., Matsuzaki, F., Iida, K., Harada, F., Kasuga, M., Sakai, H. and Yahara, I. (1986) Proc. Natl. Acad. Sci. USA 83, 8054–8058.
[13] Strickland, S., Smith, K.K. and Marotti, K.R. (1980) Cell 21, 347–355.
[14] Kondo, K., Tsuneizumi, K., Watanabe, T. and Oishi, M., manuscript submitted.
[15] Moore, S.K., Kozak, C., Robinson, E.A., Ullrich, S.J. and Appella, E. (1989) J. Biol. Chem. 24, 5343–5351.
[16] Wisnievski, J., Fronk, J. and Toczko, K. (1985) Anal. Biochem. 148, 245–267.
[17] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
[18] Cleveland, D.W., Lapata, M.A., MacDonard, R.J., Cowan, N.J., Rutter, W.J. and Kirschner, M.W. (1980) Cell 20, 90–105.
[19] Feinberg, A.P. and Vogelstein, B. (1984) Anal. Biochem. 137, 266.
[20] Laemli, U.K. (1970) Nature 227, 680–685.
[21] Strickland, S. and Mahdavi, V. (1989) Cell 15, 393–403.
[22] Uehara, Y., Hori, M., Takeuchi, T. and Umezawa, H. (1986) Mol. Cell. Biol. 6, 2198–2206.
[23] Uehara, Y., Murakami, Y., Mizuno, S. and Kawai, S. (1988) Virology 164, 294–298.
[24] Murakami, Y., Mizuno, S., Hori, M. and Uehara, Y. (1988) Cancer Res. 48, 1587–1590.
[25] Kondo, K., Watanabe, T., Sasaki, H., Uehara, Y. and Oishi, M. (1989) J. Cell Biol. 109, 285–293.